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(71) Applicant: COULTER CORPORATION [US/US]; P.O. Box 169015, Miami, FL 33116-9015 (US).

(72) Inventors: O'BRIEN, Melissa, C.; 1227 Fairlake Trace #714, Fort Lauderdale, FL 33326 (US). BOLTON, Wade, E.; 3950 S.W. 84 Terrace, Davie, FL 33328 (US).

(74) Agent: KAYE, Michelle, A.; Patent Dept. MC 32-A02, Coulter Corporation, P.O. Box 169015, Miami, FL 33116-9015 (US).

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(54) Title: VIABILITY PROBES FOR ISOLATION, IDENTIFICATION AND/OR ANALYSIS OF CELLS

(57) Abstract

The present invention provides a viability probe which can be used independently or is useful for inclusion in a monoclonal antibody panel for the analysis of solid or non-adherent tumors.

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VIABILITY PROBES FOR ISOLATION,
IDENTIFICATION AND/OR ANALYSIS OF CELLS

Technical Field

The present invention provides viability probes which
can be use independently or are useful for inclusion in a
monoclonal antibody panel for the analysis of solid or
non-adherent tumors.

Background Art

Non-viable, permeabilized cells constitute a
significant source of interference in flow cytometric
analysis. This interference results primarily from
differential antigen expression (13), biochemical changes
(e.g., increased autofluorescence) (24), and the
nonspecific uptake and binding of probes due to membrane
permeabilization accompanying cell death (6,15,20,22).
Accurate analysis of the viable cells, therefore, requires
exclusion of the non-viable cells (20).

The two best characterized processes of cell death
are necrosis and apoptosis (1,3,5,9,13,19). Necrosis is
typically induced by extracellular conditions, such as
hypoxia or cytotoxic agents, and is characterized by a
sequence of metabolic collapse, cell swelling, lysis, and
inflammation of surrounding tissue (1,5,7,9,23). The more
common phenomenon of apoptosis (1,10) is associated with
programmed cell death, and is characterized by a sequence
of cell shrinkage, chromatin condensation and cleavage,
and finally, membrane permeabilization (1,3,5,7,9,10,13).
As dying cells permeabilize, changes in their morphology
and refractive index significantly alter the cells' light
scatter characteristics, and the loss of membrane
integrity permits uptake of normally impermeant probes.

The most widely used flow cytometric dead cell
discrimination techniques can be divided into three main
categories: (1) physical separation methods, (2)
differential light scatter, and (3) differential staining
by fluorescent probes. These techniques are largely

measures of membrane permeabilization, and are based on
the equation of this phenomenon with cell death (6,13).

It is apparent, therefore, that treatment of samples
with permeabilizing agents, as is often necessary for
5   sample preservation, intracellular labelling, or biohazard
control, presents a significant challenge to the
discrimination of naturally permeabilized dead cells from
cells that were alive prior to artificial
permeabilization. Such agents can drastically alter
10   cellular morphology and refractive index, resulting in
homogenization of the light scatter characteristics of
live and dead cells, as well as cells of different types.
Moreover, permeabilizing agents allow all cells, including
those that were live prior to permeabilization, to take up
15   any probe present in excess in the solution, or that leaks
back out of previously stained dead cells. Consequently,
fixation and permeabilization generally preclude the use
of the two most common fresh prep techniques for dead cell
discrimination, i.e., electronic gating on the basis of
20   light scatter and uptake of membrane-impermeant probes.

Thus, there is both a need for discrimination of
cells that were dead prior to permeabilization and the
lack of suitable techniques for doing so. Physical
separation methods such as percoll and ficoll density
25   gradient separation can separate dead from live cells on
the basis of differing buoyant densities (5), but have the
disadvantages of wide variations in purity and recovery,
selective cell loss (11), and in some circumstances, cell
toxicity (21). Light scatter-based dead cell
30   discrimination is often inapplicable to heterogeneous cell
mixtures such as solid tumors, because dead cells of one
type may have light scatter characteristics similar to
those of live cells of another type (15,20). Lastly, few
of the known fluorescent viability probes (e.g., propidium
35   iodide, fluorescein diacetate, etc.) are considered
compatible with the use of permeabilizing agents,
primarily due to weak or reversible binding, allowing them

to leach out of stained cells and into artificially permeabilized unstained cells (15,20).

Pollack et al. have reported successful dead cell discrimination using PI in ethanol-fixed samples (14).

5   The present invention provides viability probes for inclusion in solid tumor monoclonal antibody panels, which established the following criteria: broad cell type specificity; low nonspecific staining of live cells; high signal-to-noise staining of dead cells; binding in dead

10   cells of sufficiently high affinity to withstand the permeabilization procedure; and excitation and emission spectra compatibility with the other dyes in the solid tumor panel.

Disclosure of the Invention

15   Accordingly, it is an object of the subject invention to provide viability probes which are useful for inclusion in a monoclonal antibody panel for the analysis of solid, preferably breast, tumors.

It is another object of the present invention to

20   provide a viability probe directed against a relatively stable intracellular antigen.

It is an additional object of the present invention to provide a viability probe that provides information regarding late apoptotic and necrotic cells containing

25   degraded chromatin (Type III).

It is an additional object of the present invention to provide a viability probe that provides information regarding permeabilized cells containing native chromatin (Type I).

30   It is another object of the present invention to provide a viability probe that has the significant advantage of being resistant to fixation and permeabilization procedures, such as are often required for sample preservation, biohazard control, or

35 intracellular labelling.

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It is another object of the present invention to provide an antibody probe which can be conjugated to any of a wide array of available fluorophores.

5   The viability probes of the present invention may be use in the methods of application having docket number 139,646, entitled "Solid Tumor Analysis by Multiparametric Flow Cytometry", filed concurrently herewith. Said application is herein incorporated by reference.

Additional objects, advantages and novel features of
10   the invention will be set forth in part in the description which follows and in part will become apparent to those skilled in the art upon examination of the following or may be learned by practice of the invention. The objects and advantages of the invention may be realized and
15   attained by means of the instrumentalities and combinations particularly pointed out in the appended claims.

Brief Description of Drawings

The preferred embodiments of this invention will now
20   be described by way of example, with reference to the drawings accompanying this specification in which:

FIG. 1 shows a sample processing flow chart;

FIG. 2 shows a comparison of light scatter (LT SCATTER) and PI staining of unprocessed cells with
25   staining of processed cells by experimental viability probes actin-SAM-FITC (ACTIN-SF), cytokeratin-SAM-FITC (CK-SF), 7-AAD, and TO-PRO-3.

FIG.3 shows a comparison of light scatter (LT SCATTER) and PI staining of unprocessed cells (PI UNFIXED)
30   with staining of processed cells by experimental viability probes LDS-751 (LDS), tubulin-SAM-FITC (TUBULIN-SF), EMA, and PI (PI FIX).

Modes for Carrying Out the Invention

The occurrence of often large apoptotic and necrotic
35   populations common to solid tumors presents a significant

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obstacle to the accurate flow cytometric analysis of tumor
cells. Indeed, it is quite possible, particularly in
large tumors or stored specimens, for live tumor cells to
comprise only a small minority of the total cell
5   population. Unless the live tumor cells can be identified
and isolated, the potential for erroneous immunophenotypic
and DNA analysis may be prohibitive, due to wide
variations in autofluorescence background of different
cell types and dead cells, nonspecific cell surface
10   antibody binding, and nonspecific antibody uptake, and
chromatin degradation in dead cells.

The present invention provides viability probes which
are useful for inclusion in a monoclonal antibody panel
for the analysis of solid, preferably breast, tumors.

15   Applicants have discovered that the viability probes
of the present invention demonstrate sufficiently broad
cell type specificity to be informative for the large
variety of cells found in solid tumors (4); low
nonspecific binding; specific binding strong enough to
20   withstand the staining and permeabilization procedures;
both high and stable fluorescence intensity, to remain
informative over the period of time dead cells may persist
in a solid tumor before disintegration (at which point the
debris can be discriminated or gated out on the basis of
25   light scatter); and compatibility with the four other
fluorescent probes comprising the tumor antibody panel.

Applicants have found that tubulin-SAM-FITC appears
to satisfy all of the aforementioned criteria: the
presence of tubulin in all eukaryotic cells (18)
30   circumvents the problem of tumor heterogeneity; the probe
exhibits little or no nonspecific binding to intact cells,
and its binding in permeabilized cells is sufficiently
strong to withstand the staining and permeabilization
procedures; its signal-to-noise ratio is high enough to
35   clearly distinguish it from autofluorescence even through
day 15 after cell harvest, and its fluorescence intensity
diminishes relatively slowly over the time course; and it

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is compatible with the other probes comprising the tumor
antibody panel. Cytokeratin-SAM-FITC was satisfactory in
every respect other than its specificity only for
epithelial cells. Actin, EMA, and 7-AAD did not display
5 sufficiently high signal-to-noise ratios over the entire
time course. TO-PRO-3, LDS-751, and PI (processed cells)
similarly stained both live and dead cells, and are
therefore uninformative for viability in this application.
       Recent reports that membrane permeabilization pre-
10 cedes chromatin degradation in necrosis and follows it in
apoptosis (1,5,7,13) imply the occurrence of a least three
significant types of dead cells, described in Table 1.

Table 1
Types, Characteristics, and Detection of Dead Cells

DEAD CELL TYPE	STAGE OF CELL DEATH	CHARACTERISTICS	VIABILITY PROBE DETECTION
I	Early necrosis	Native chromatin/ permeable cell membrane	DNA-specific dyes compatible with fixation/arti- ficial permeabil- ization; anti- tubulin
II	Early apoptosis	Degraded chromatin/ intact cell membrane	None compatible with fixation/ artificial permeabilization
III	Late necrosis/ Late apoptosis	Degraded chromatin/ permeable cell membrane	anti-tubulin

       The present invention supports this categorization.
The existence of Types I and III dead cells is suggested
by the fact that staining by DNA-specific probes is most
intense at the onset of cell death and decreases with
25 increasing cell age after harvest. The existence of Type
II dead cells, with intact membranes and degraded
chromatin, is predicted by the consistently higher
percentages (Tables 2 and 3) of cells with low forward

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scatter (suggesting the loss of cell volume that precedes chromatin degradation in apoptosis) (7) than positivity for the viability probes (excluding TO-PRO-3, LDS-751, and PI in processed cells, all of which stained live and dead
5   cells indiscriminately).

The viability probes of the present invention are further directed against a relatively stable intracellular antigen which proves more informative than traditional, DNA-specific viability dyes (e.g., propidium iodide) for
10   late apoptotic and necrotic cells containing degraded chromatin (Type III), as well as permeabilized cells containing native chromatin (Type I). The present invention has the significant advantage of being resistant to fixation and permeabilization procedures, such as are
15   often required for sample preservation, biohazard control, or intracellular labelling. Finally, the present invention teaches the use of an antibody probe, which can be conjugated to any of a wide array of available fluorophores, which has broader application potential than
20   more traditional dye probes of fixed, and frequently broad, emission spectra.

In a preferred embodiment of the present invention the viability probe is cytokeratin. In the most preferred embodiment of the present invention the
25   viability probe is tubilin.

The present invention provides two time-course studies presented below, wherein tumor cell lines were induced to enter a condition of cellular hypoxia intended to simulate the internally hypoxic environment of a solid
30   tumor, and in which their degree of staining by numerous viability probes is compared. The first study involved staining of the MDA (breast tumor) cell line by propidium iodide (PI) without permeabilization as the standard, and, with permeabilization, anti-actin-SAM-FITC, anti-
35   cytokeratin-SAM-FITC, 7-aminoactinomycin D (7-AAD; a high-affinity DNA intercalator with an emission maximum at approximately 650 nm) (16,17), and TO-PRO-3 (a membrane-

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impermeant, high-affinity DNA intercalator with an
emission maximum at 661 nm, and minimal fluorescence
except when bound to nucleic acids). The second study
included staining of MDA cells by PI without
5   permeabilization as the standard, and, with
permeabilization, anti-tubulin-SAM-FITC, ethidium
monoazide bromide (EMA, a photoactivated, covalently
binding DNA intercalator with an emission maximum at
approximately 600 nm) (15), LDS-751 (a vital nucleic acid
10 stain with an emission maximum at 670 nm) (20), and PI.

Materials
        PBS, lysophosphatidyl choline, Nonidet P-40 (NP40),
trypan blue, anti-α-smooth muscle actin monoclonal
antibody, and 7-aminoactinomycin D (7-AAD) were obtained
15 from Sigma Chemical Co. (St. Louis, MO). FBS and absolute
methanol were purchased from HyClone (Logan, UT) and JT
Baker, Inc. (Phillipsburg, NJ), respectively. Propidium
iodide (PI) stain (from the DNA-Prep Kit; contains 50
ug/ml PI, 4 KU/ml bovine pancreas Type III RNAse, 0.1%
20 NaN_3, saline, and stabilizers) and anti-cytokeratin
monoclonal antibody were provided by Coulter Corporation
(Miami, Florida). Anti-α-tubulin was purchased from Zymed
Laboratories, Inc. (San Francisco, CA). FITC-conjugated
sheep-anti-mouse F(ab') fragments (SAM-FITC) were obtained
25 from Silenus Laboratories (Victoria, Australia). Ethidium
monoazide bromide (EMA) and TO-PRO-3 were from Molecular
Probes, Inc. (Eugene, OR). LDS-751 was purchased from
Exciton (Dayton, OH).

Cell Culture
30   Monolayer cultures of the MDA-MB-175-VII breast
cancer cell line (American Type Culture Collection,
Rockville, MD) were grown in high-glucose DMEM
(Biowhittaker, Walkersville, MD) supplemented with 10%
fetal bovine serum (HyClone, Logan, UT) at 37°C and 5% CO_2
35 in a humidified atmosphere. The cultures were maintained

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in log phase growth prior to harvesting. For each of the
time-course studies presented here, cells were harvested
daily (excluding weekends) and subsequently held, tightly
capped, at 37° and 5% CO_2 in a humidified atmosphere, to
5   simulate intratumoral hypoxia. In each study, upon
harvest of the twelfth daily batch of cells, samples from
each batch were processed for flow cytometric analysis.

Cell Staining
        For purposes of evaluating viability probes for
10   inclusion in our solid tumor monoclonal antibody panel,
samples were processed according to the procedure used in
our laboratory for simultaneous surface and intranuclear
staining of solid tumors.
        In each of the time-course studies, after the last of
15   twelve daily (excluding weekends) batches of cells was
harvested, aliquots were taken from each batch for trypan
blue viability staining and microscopic enumeration.
Cells from each batch were then aliquoted, at 1×10^6 cells
per test, into 12 x 75 mm siliconized glass test tubes.
20   In each experiment, the samples were divided into two
groups: an unprocessed (i.e., not processed by the solid
tumor staining procedure below) series to be stained with
propidium iodide (PI) as a standard, and a series to be
stained with other viability dyes and processed according
25   to the solid tumor staining procedure below (Fig. 1).
Thus, in Study 1, for each day's harvest of MDA cells (day
0, the youngest cells, through day 15, the oldest cells),
there were seven samples: an unprocessed, unstained
control; an unprocessed, PI-stained standard; a processed,
30   unstained control; and four processed samples stained with
either actin-SAM-FITC, cytokeratin-SAM-FITC, 7-AAD, or TO-
PRO-3. In Study 2, there were also seven samples for each
day's harvest of MDA cells: an unprocessed, unstained
control; an unprocessed, PI-stained standard; a processed,
35   unstained control; and four processed samples stained with
either tubulin-SAM-FITC, EMA, LDS-751, or PI. It should

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be noted that unstained cells, rather than isotype
controls, were used here to establish fluorescence
background for antibody-labelled cells. The intensity of
antibody staining inside a cell is the result of a

5   combination of factors, including specific binding to the
target antigen, nonspecific antibody binding, antibody
entrapment, and nonspecific binding of the dye itself.
Clearly, when the measurement of specific binding to a
target antigen is the objective, isotype controls must be

10 used to estimate the level of nonspecific fluorescence.
However, when, as in this case, the objective is merely to
distinguish permeabilized from intact cells by fluorescent
antibody uptake, the more antibody taken up (whether
specifically or nonspecifically), the better the

15 distinction. Since the determining factor in this
application was total, rather than specific, fluorescence,
isotype controls were unnecessary.

    After aliquoting the cells, the unprocessed series to
be stained with PI and their unstained controls were

20 centrifuged at 500 x g for 5 minutes at room temperature
and decanted. The unstained controls were resuspended in
PBS supplemented with 2.5% FBS (PBSF), and the PI-stained
samples were resuspended in 1 ml and incubated in darkness
at room temperature for twenty minutes prior to flow

25 cytometric analysis.

    The remaining samples were centrifuged as above and
decanted. Unstained controls and cells to be stained with
7-AAD, TO-PRO-3, LDS-751, EMA, or PI were resuspended in
200 μl PBSF and incubated for 15 minutes at room

30 temperature. The remaining samples were resuspended in
200 μl each of either a 1:200 dilution of actin in PBSF
(protein concentration unavailable), a 1:200 dilution of
tubulin in PBSF (protein concentration unavailable), or 25
μg/ml cytokeratin in PBSF (optimal doses for all three

35 antibodies were previously established by titration; data
not shown), and incubated for 15 minutes at room
temperature. All samples were then washed with 2 ml PBS

each, centrifuged as above, and decanted. Unstained
controls were then resuspended in 200 µl PBSF and
incubated for 15 minutes at room temperature. Samples
stained with actin, cytokeratin, or tubulin were

5   resuspended in 200 µl of 48.8 µg/ml SAM-FITC in PBSF and
incubated in darkness for 15 minutes at room temperature.
7-AAD-stained cells were resuspended in 1 ml/test of 25
µg/ml 7-AAD (250 µl of stock solution in 49.75 ml PBSF;
stock solution is 5 mg/ml in methanol) and incubated in

10   darkness for 15 minutes at room temperature. TO-PRO-3-
stained cells were resuspended in 1 ml/test of 5 µM TO-
PRO-3 in PBSF and incubated in darkness for 15 minutes at
room temperature. LDS-751-stained cells were resuspended
in 1 ml/test of PBSF containing 10 µl of 2 µg/ml LDS-751

15   (50 µl of stock solution in 4.95 ml PBSF; stock solution
is 0.2 mg LDS-751/ml methanol) and incubated in darkness
for 15 minutes at room temperature. EMA-stained cells
were resuspended in 10 µl/test of 5 µg/ml EMA (125 µl of
stock solution in 4.875 ml PBSF; stock solution is 0.2 mg

20   EMA/ml methanol) and incubated for 15 minutes at room
temperature, at a distance of 20 cm from a fluorescent
light bulb. PI-stained cells were resuspended in 1
ml/test of the same PI solution used for unprocessed cells
and incubated in darkness for 15 minutes at room

25   temperature. All samples were then washed as above,
followed by resuspension in 200 µl/test PBSF, and a 15-
minute, room-temperature incubation in darkness (to
simulate surface antigen staining of tumor samples). All
samples were then washed twice more as above. After

30   decanting the supernatant, each sample was resuspended in
1 ml of 25°C 20 µg/ml lysophosphatidyl choline in 1%
paraformaldehyde, incubated for 2 minutes at room
temperature, centrifuged at 500 x g for 5 minutes at room
temperature, and decanted. The samples were then

35   resuspended in 1 ml/test of -20°C absolute methanol and
incubated on ice for 10 minutes, followed by
centrifugation as above. After decanting, the samples

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were resuspended in 1 ml/test of 4°C 0.1% NP40 and
incubated on ice for 5 minutes, followed by centrifugation
as above. After decanting, samples were resuspended in
200 μl/test of PBSF and incubated in darkness for 15
5   minutes at room temperature (to simulate intracellular
antigen staining of tumor samples), followed by washing as
above. Finally, the samples were resuspended in 1 ml/test
PBSF prior to flow cytometric analysis.

Flow Cytometry
10   Samples were analyzed on an EPICS® Elite flow
cytometer (Coulter Corporation, Miami, FL) configured with
five photomultiplier tubes and three lasers: a water-
cooled, 5 W argon laser, an air-cooled 488 nm argon laser
operated at 15 mW, and a 10 mW air-cooled 633 nm helium-
15   neon laser. TO-PRO-3 stained samples were analyzed with
633 nm excitation; all other samples were analyzed with
488 nm excitation by the air-cooled argon laser.
Fluorescence emission of actin-, cytokeratin-, and
tubulin-SAM-FITC was reflected by a 550 nm dichroic long-
20   pass filter and passed through a 525 nm bandpass filter.
PI and EMA fluorescence were reflected by a 650 nm
dichroic long-pass filter and passed through a 610 nm
bandpass filter. 7-AAD, TO-PRO-3, and LDS-751
fluorescence passed through 650 nm dichroic long-pass and
25   675 nm bandpass filters. Autofluorescence of unstained
controls was measured through the same filter
configurations as the stained samples with which they were
matched.
          Five thousand events were collected in listmode
30   format for each sample, with linear amplification of
forward and side scatter, and four-decade logarithmic
amplification of fluorescence. Acquisition and analysis
were performed with the Elite software; color graphics
were performed with WinList software (Verity Software
35   House, Topsham, Maine) and Excel® software (Microsoft
Corporation, Redmond, WA).

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The results of the first time-course study are
presented in FIG. 2 and Table 2. In FIG. 2, the leftmost
column indicates the number of days the batch of cells in
the corresponding row was held after harvest (days 5, 6,
5   12 and 13 coincided with weekends, during which cells were
not harvested). The next column displays light scatter
results for unprocessed cells from each batch (in each of
these histograms, forward scatter is on the Y axis, and
side scatter is on the X axis). The remaining columns
10   display fluorescence histograms of unstained cells (in
green) electronically overlaid with histograms (in colors
other than green) of cells stained with the probe
indicated at the top of each column. All instrument
settings remained unchanged for the duration of the
15   experiment. Fluorescence histograms are on a four-decade
logarithmic scale. All histograms contain 5,000
autoscaled events.

In general, dead cells exhibit a decrease in forward
angle light scatter, an increase in 90° angle light
20   scatter, and an increase in fluorescence when stained
(prior to or in the absence of artificial
permeabilization) with fluorescent probes (17). Thus, to
satisfy our criteria, a viability probe should display, in
FIGS. 2 and 3, three characteristics: 1) little or no
25   staining of live cells (i.e., the non-green peaks of the
test samples in the first row of FIGS. 2 and 3 should
match the green peaks of the unstained controls they
overlay as closely as possible); 2) fluorescence
bimodality comparable to the proportion of live and dead
30   cells in the sample, as indicated in FIGS. 2 and 3 by
light scatter (i.e., the non-green peaks of the test
samples should be bimodal in rows [primarily days 1-4] in
which the light scatter histogram indicates the presence
of both live and dead cells, and should be unimodal in
35   rows [primarily days 0 and 7-15] in which the light
scatter histogram indicates the presence of only live or
only dead cells); and 3) fluorescent staining of dead

Table 2

Study 1: Percent Positivity and Mean Channel Fluorescence Comparison of Experimental Viability Probes

DAY	LS %POS[a]	PI (Unprocessed Cells) %POS[b]	MC[c]	ACTIN-SAM-FITC %POS	MC	CYTOKERATIN-SAM-FITC %POS	MC	7-AAD %POS	MC	TO-PRO 3 %POS	MC
0	13.2	6.8	768.4	6.6	14.4	9.1	46.3	4.2	9.86	100	264.8
1	69.5	52.3	903.2	49.8	21.4	54.2	538.9	43.9	12.4	100	154.3
2	73.4	61.3	611.4	55.8	15.2	57.4	377.3	54.1	11.0	100	144.7
3	82.8	65.7	409.8	68.1	22.0	68.8	319.9	67.8	9.44	100	119.2
4	91.8	68.3	263.6	75.5	14.1	75.5	289.7	-	3.68	100	74.9
7	100	82.3	106.1	95.6	8.04	88.0	172.9	-	3.70	100	42.4
8	100	86.7	68.6	96.2	8.85	88.3	215.9	-	3.77	100	32.8
9	100	85.3	62.2	94.7	11.4	86.2	192.6	-	3.88	100	38.0
10	100	87.3	47.3	94.5	8.58	90.1	178.5	-	2.86	100	30.7
11	100	86.2	43.3	91.0	11.9	82.4	170.7	-	3.12	100	28.6
14	100	88.8	36.7	93.2	15.7	84.7	203.6	-	4.49	100	25.3
15	100	84.7	32.3	94.4	9.83	-	95.9	-	3.26	100	20.4

[a] LS %POS refers to the percentage of cells displaying the low forward scatter characteristic of dead cells

[b] %POS refers to percent positivity

[c] MC refers to mean channel (unconverted values from a 4-decade logarithmic scale)

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cells that is significantly brighter than the
autofluorescence of the unstained controls (i.e., the
positive non-green peaks of the test samples should be as
far apart as possible from the green peaks of the
5   unstained controls they overlay). It should be emphasized
that the green peaks in each fluorescence histogram
correspond to unstained controls (electronically overlaid
with stained samples in colors other than green), not
negatively stained cells.

10   In FIG. 2, nonspecific staining of live cells was
minimal for actin-SAM-FITC and cytokeratin-SAM-FITC, dim
for 7-AAD, relatively bright for PI, and very bright for
TO-PRO-3. The fluorescence distributions of PI, actin-
SAM-FITC, cytokeratin-SAM-FITC, and 7-AAD corresponded to
15   light scatter and trypan blue uptake (data not shown) in
the relative proportions of their negative (live cell) and
positive (dead cell) peaks; TO-PRO-3's fluorescence
distribution remained unimodal throughout the time course.
Of the three probes -- actin-SAM-FITC, cytokeratin-SAM-
20   FITC, and 7-AAD -- suitable for this application,
cytokeratin-SAM-FITC exhibits the highest signal-to-noise
ratio, remaining distinct from autofluorescence through
day 15. By day 4, neither actin nor 7-AAD staining is
significantly distinct from autofluorescence. Neither PI,
25   due to its reversible binding, nor TO-PRO-3, because of
its intense staining of both live and dead cells without
significant distinction, are acceptable as viability
probes for this application.

In Table 2, the percentages of cells stained
30   positively by the various viability probes used in Study 1
are compared with the percentages of cells displaying the
low forward and high side scatter characteristic of dead
cells. Because certain probes stained live cells
nonspecifically, percentage positivity of staining was
35   calculated relative to the negative peak in bimodal
distributions, rather than to unstained cells. At the
point in each column where bimodality was completely lost,

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a dash appears in the table. Interestingly, the light
scatter-based percentages are consistently and
significantly higher than the fluorescence-based
percentages. PI, actin-SAM-FITC, cytokeratin-SAM-FITC,
5   and 7-AAD yield comparable positivity through day 4, when
the bimodality of the 7-AAD distribution is lost. PI,
actin, and cytokeratin positivity remain comparable
through day 15 after harvest. This finding is
significant, because it suggests that the much greater
10   size of the antibody sandwich complexes, relative to the
DNA-specific dyes, does not impede staining. The unimodal
distribution of TO-PRO-3 throughout the study, and its
intense staining of live cells, make it uninformative here
for viability. Mean channel values of fluorescence
15   intensity are also reported. In general, after day 0,
when staining is minimal (except for TO-PRO-3) due to the
preponderance of live cells, the fluorescence intensity of
all the probes decreases, though at different rates. PI
and TO-PRO-3 fluorescence intensities show the steepest
20   decline, followed by cytokeratin and, in tandem, actin and
7-AAD. Conversely, autofluorescence (green histograms)
increases steadily, particularly in the green region of
the spectrum, with cell age after harvest.
        The results of the second time-course study are
25   presented in FIG. 3 and Table 3. The formats of both are
the same as those of FIG. 2 and Table 2, respectively.
The light scatter, autofluorescence, and PI staining of
unprocessed cells are similar to those in FIG. 2. In FIG.
3, nonspecific staining of live cells was minimal for
30   tubulin-SAM-FITC, and relatively high for PI (in both
processed and unprocessed cells), LDS-751, and EMA. The
fluorescence distributions of PI in unprocessed cells,
tubulin-SAM-FITC, and EMA corresponded to light scatter
and trypan blue uptake in the relative proportions of
35   their negative and positive peaks; the fluorescence
distributions of PI in processed cells and LDS-751

Table 3

Study 2: Percent Positivity and Mean Channel Fluorescence Comparison of Experimental Viability Probes

DAY	LS %POS[a]	PI (Unprocessed Cells)		LDS-751		TUBULIN-SAM-FITC		EMA		PI (Processed Cells)	
		%POS[b]	MC[c]	%POS	MC	%POS	MC	%POS	MC	%POS	MC
0	14.0	5.9	483.0	98.2	12.4	6.2	10.3	4.2	12.4	100.0	6.42
1	59.8	40.7	547.2	97.7	31.2	36.6	273.1	31.6	19.7	97.1	29.8
2	76.7	51.0	358.4	93.7	20.2	56.9	383.2	49.9	16.7	93.3	31.7
3	85.2	66.2	242.1	87.2	21.4	67.1	330.3	58.6	15.9	91.0	27.6
4	88.7	70.6	155.8	88.4	53.5	74.7	339.1	71.6	16.6	93.1	10.8
7	100	87.4	67.1	88.8	72.0	81.8	323.8	84.4	13.2	92.5	4.12
8	100	86.8	41.0	84.1	66.5	82.4	343.9	86.6	16.9	90.3	3.77
9	100	89.4	31.5	95.3	40.5	86.4	328.3	87.9	14.8	96.0	3.63
10	100	89.3	51.5	90.8	66.9	89.2	205.2	88.8	13.5	94.4	5.08
11	100	89.8	25.3	86.7	68.0	90.0	188.2	90.1	12.0	91.3	3.70
14	100	89.4	15.8	89.5	61.2	85.0	272.2	90.2	12.4	91.5	4.02
15	100	88.2	15.1	83.9	58.4	88.6	284.0	90.2	12.8	91.9	4.43

[a] LS %POS refers to the percentage of cells displaying the low forward scatter characteristic of dead cells

[b] %POS refers to percent positivity

[c] MC refers to mean channel (unconverted values from a 4-decade logarithmic scale)

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remained essentially unimodal throughout the time course.
Of the two probes -- tubulin-SAM-FITC and EMA -- suitable
for this application, tubulin-SAM-FITC exhibits the
highest signal-to-noise ratio, remaining very distinct
5 from unstained cells through day 15. By day 7, the
difference between EMA staining and autofluorescence is
approximately the same as the level of nonspecific
staining in live cells at day 0. Neither PI, due to its
reversible binding, nor LDS-751, because of its
10 consistently unimodal fluorescence distribution, are
acceptable as viability probes for this application.

        As in Table 2, the light scatter-based percentages of
dead cells in Table 3 are consistently and significantly
higher than the fluorescence-based percentages. PI in
15 unprocessed cells, tubulin-SAM-FITC, and EMA yield
comparable positivity throughout the time course. The
consistently unimodal distributions of LDS-751 and PI in
processed cells make them uninformative here for
viability. The mean fluorescence intensity of PI in both
20 processed and unprocessed samples declines rapidly over
the time course, while that of EMA and tubulin-SAM-FITC
diminishes slowly. Interestingly, the fluorescence
intensity of LDS-751 appears to be cyclical over the time
course, while all of the other probes exhibit a linear
25 decrease in fluorescence intensity.

        All publications cited in this specification are
indicative of the level of skill of those in the art to
which this application pertains. Each publication is
individually incorporated herein by reference in the
30 location where it is cited.

        One skilled in the art will appreciate that although
specific reagents and conditions are outlined in the above
preparations and methods, modifications can be made which
are meant to be encompassed by the spirit and scope of the
35 invention. The preparations and methods, therefore, are
provided to illustrate the invention. Such alternate
means are to be construed as included within the intent

and spirit of the present invention as defined by the following claims.

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Claims

1.   A method of discriminating dead cells in a
heterogeneous biological sample which method is
characterized by:
5   (a) staining cells with a viability probe said probe
being resistant to fixation and permeabilization
procedures; and
         (b) analyzing by a fluorescence measuring instrument.

2.   The method according to claim 1 further characterized
10 by the viability probe which is a nucleic acid specific
dye.

3.   The method according to claim 1 further characterized
by the viability probe which is an intracellular antigen.

4.   The method according to claim 3 further characterized
15 by the intracellular antigen which is tubulin.

5.   The method according to claim 1 further characterized
by the viability probe which is a cytoskeletal antigen.

6.   The method according to claim 5 further characterized
by the cytoskeletal antigen which is cytokeratin.

20 7.   The method of claim 1 further characterized by the
cells which are neoplastic cells.

8.   The method of claim 7 further characterized by the
neoplastic cells which are solid tumor cells.

9.   The method of claim 7 further characterized by the
25 neoplastic cells which are breast tumor cells.

10.   The method of claim 1 further characterized by the
cells which are non-adherent tumor cells.

11.   The method of claim 10 further characterized by the
non-adherent tumor cells which are hematopoietic
neoplasias.

12.   A method of discriminating late apoptotic cells
5 containing degraded chromatin (Type III) which method is
characterized by:
       (a)   staining cells with a viability probe, said
probe being resistant to fixation and permeabilization
procedures; and
10   (b) analyzing by flow cytometry.

13.   A method of discriminating necrotic cells containing
degraded chromatin (Type III) which method is
characterized by:
       (a) staining cells with a viability probe, said probe
15 being resistant to fixation and permeabilization
procedures; and
       (b) analyzing by flow cytometry.

14.   A method of discriminating permeabilized cells
containing native chromatin (Type I) which method is
20 characterized by:
       (a) staining cells with a viability probe, said probe
being resistant to fixation and permeabilization
procedures; and
       (b) analyzing by flow cytometry.

25 15.   A method of discriminating dead cells in a
heterogeneous biological sample which method is
characterized by:
       (a) staining cells with a viability probe, said probe
being resistant to fixation and permeabilization
30 procedures;
       (b) staining with an antibody probe; and
       (c) analyzing by a fluorescence measuring instrument.

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16.   The method according to claim 15 further characterized by the viability probe which is a nucleic acid specific dye.

17.   The method according to claim 15 further
5   characterized by the viability probe which is an intracellular antigen.

18.   The method according to claim 17 further characterized by the intracellular antigen which is tubulin.

10   19.   The method according to claim 15 further characterized by the viability probe which is a cytoskeletal antigen.

20.   The method according to claim 19 further characterized by the cytoskeletal antigen which is
15   cytokeratin.

21.   The method of claim 15 further characterized by the cells which are neoplastic cells.

22.   The method of claim 21 further characterized by the neoplastic cells which are solid tumor cells.

20   23.   The method of claim 21 further characterized by the neoplastic cells which are breast tumor cells.

24.   The method of claim 15 further characterized by the cells which are non-adherent tumor cells.

25.   The method of claim 24 further characterized by the
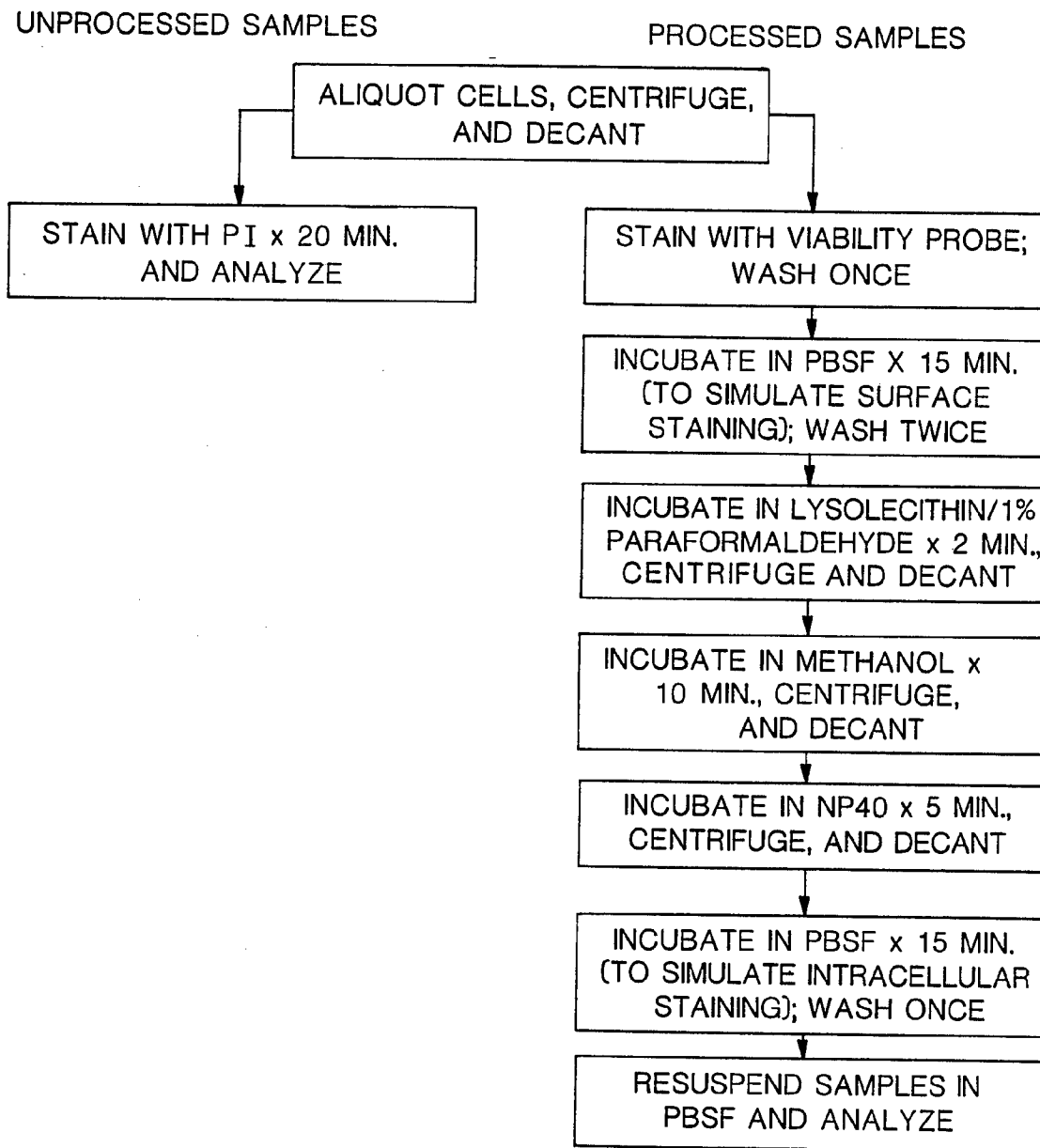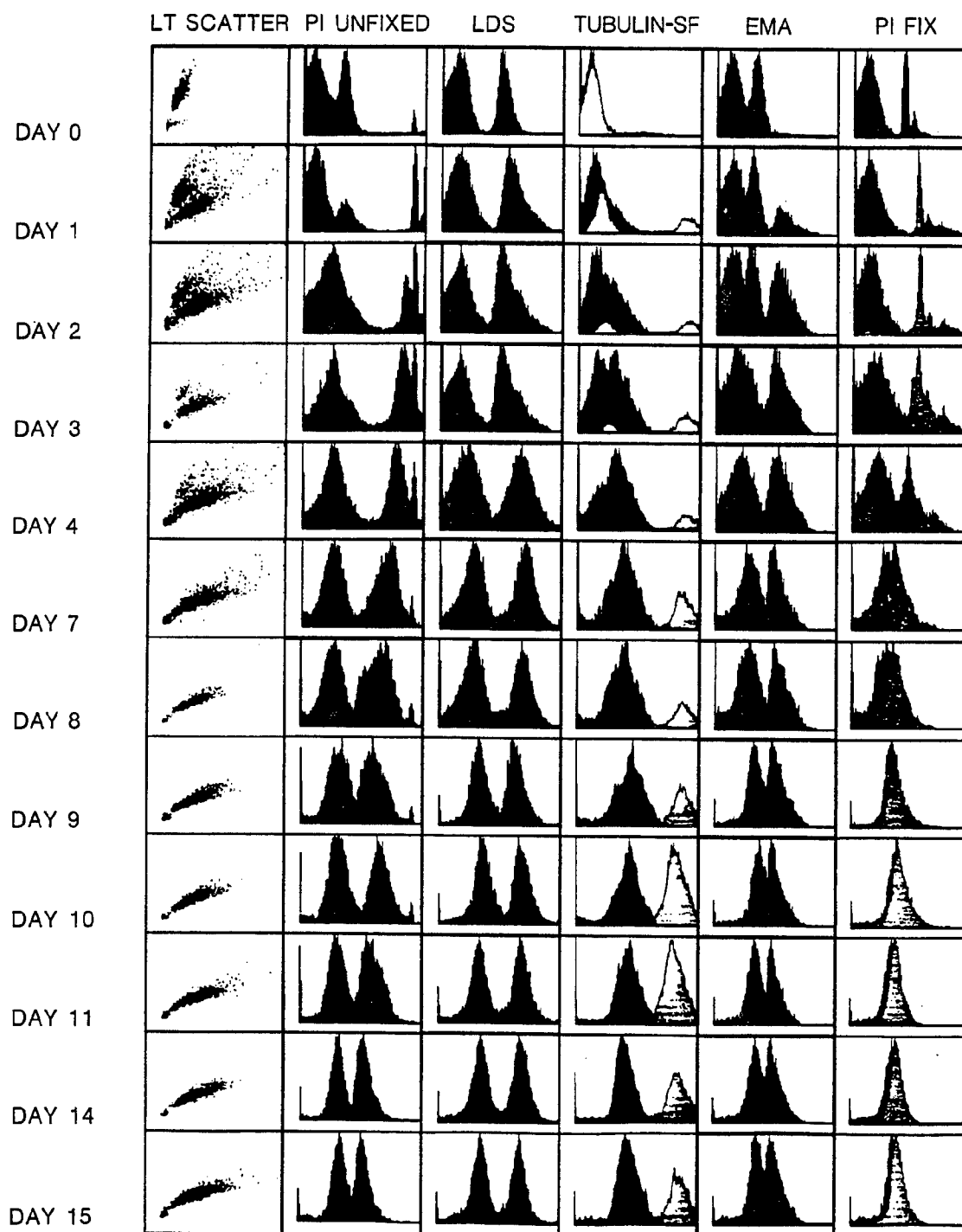25   non-adherent tumor cells which are hematopoietic neoplasias.
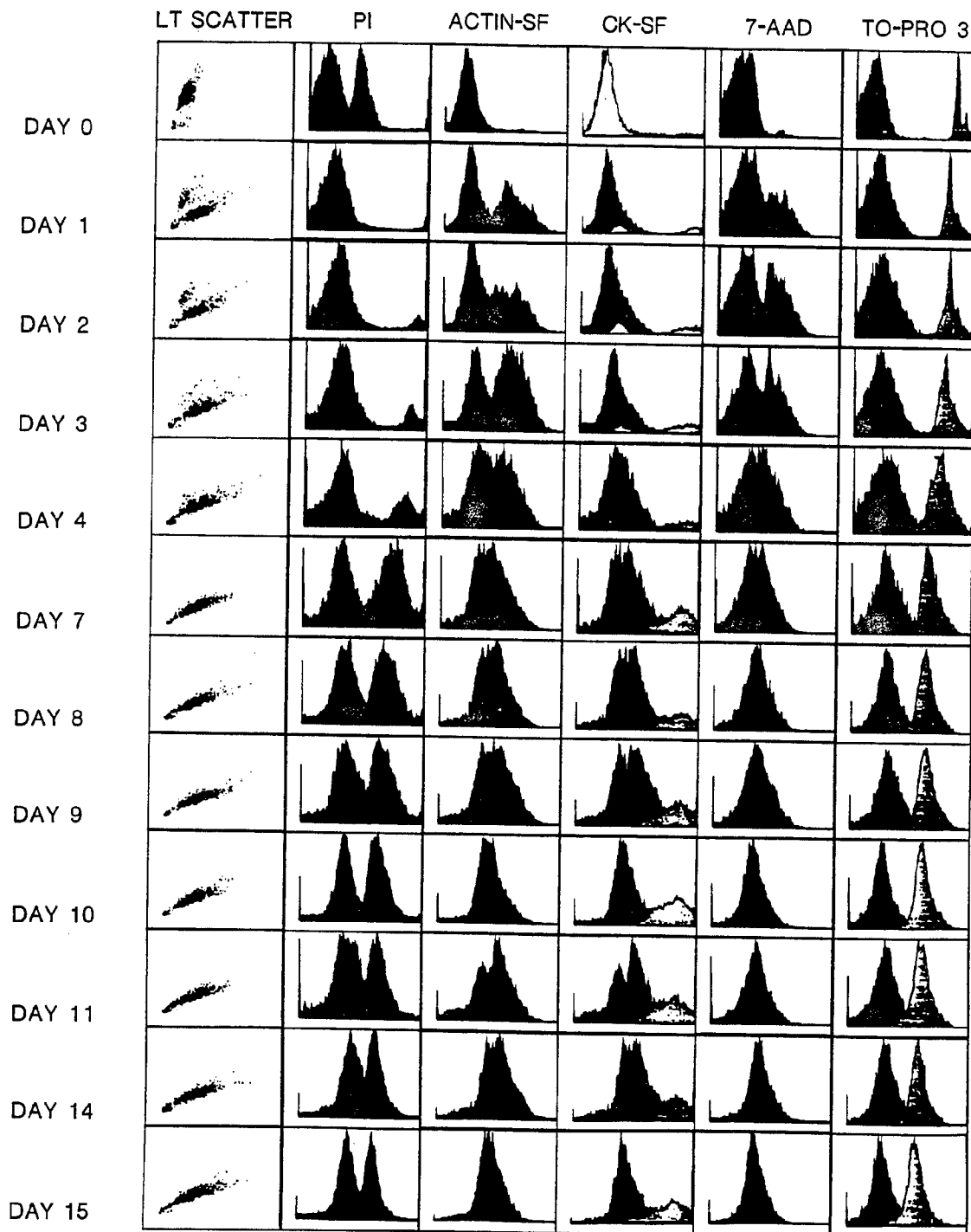
UNPROCESSED SAMPLES   PROCESSED SAMPLES

ALIQUOT CELLS, CENTRIFUGE,
AND DECANT

STAIN WITH P I x 20 MIN.
AND ANALYZE

STAIN WITH VIABILITY PROBE;
WASH ONCE

INCUBATE IN PBSF X 15 MIN.
(TO SIMULATE SURFACE
STAINING); WASH TWICE

INCUBATE IN LYSOLECITHIN/1%
PARAFORMALDEHYDE x 2 MIN.,
CENTRIFUGE AND DECANT

INCUBATE IN METHANOL x
10 MIN., CENTRIFUGE,
AND DECANT

INCUBATE IN NP40 x 5 MIN.,
CENTRIFUGE, AND DECANT

INCUBATE IN PBSF x 15 MIN.
(TO SIMULATE INTRACELLULAR
STAINING); WASH ONCE

RESUSPEND SAMPLES IN
PBSF AND ANALYZE

FIG. I

FIG. 3

FIG. 2

INTERNATIONAL SEARCH REPORT

A.	**CLASSIFICATION OF SUBJECT MATTER**

IPC(6)   :G01N 21/64, 33/49, 33/533, 33/536, 33/574
US CL   :435/2, 6, 7.23, 7.24; 436/64, 172, 546
According to International Patent Classification (IPC) or to both national classification and IPC

B.	**FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/2, 6, 7.23, 7.24; 436/64, 172, 546

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE
search terms: viability, dead, tubulin, cytokeratin, neoplastic, breast, cancer, flow cytometry

C.	**DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Cytometry, Volume 9, issued 1988, L.W.M.M. Terstappen et al, "Discriminating Between Damaged and Intact Cells in Fixed Flow Cytometric Samples", pages 477-484, see entire document.	1, 2, 12-16 ----------- 3-11, 17-25
X ---- Y	Cytometry, Volume 12, issued 1991, M.C. Riedy, et al, "Use of a Photolabeling Technique to Identify Nonviable Cells in Fixed Homologous or Heterologous Cell Populations", pages 133-139, see entire document.	1, 2, 12-16 ----------- 3-11, 17-25
Y	K. D. Bauer et al, "Clinical Flow Cytometry. Principles and Application", published 1993 by Williams and Wilkins (Baltimore, MD), pages 157-175, see pages 167-172.	1-9, 12-23

X	Further documents are listed in the continuation of Box C.		See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 APRIL 1995	03MAY1995

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	NANCY J. PARSONS
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochimica et Biophysica Acta, Volume 1133, issued 1992, C. Dive et al, "Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry", pages 275-285, see entire document.	10, 11, 24, 25